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The Hepcidin-Binding Site on Ferroportin Is Evolutionarily Conserved

Ivana De Domenico,[1,3] Elizabeta Nemeth,[2] Jenifer M. Nelson,[1] John D. Phillips,[3] Richard S. Ajioka,[3] Michael S. Kay,[4] James P. Kushner,[3] Tomas Ganz,[2] Diane M. Ward,[1] and Jerry Kaplan[1,*]
[1]Department of Pathology, School of Medicine, University of Utah, Salt Lake City, UT 84132, USA
[2]Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA 90095,
[3]Division of Hematology and Bone Marrow Transplantation
[4]Department of Biochemistry
University of Utah, Salt Lake City, UT 84132, USA
*Correspondence: jerry.kaplan@path.utah.edu
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SUMMARY

Mammalian iron homeostasis is regulated by the interaction of the liver-produced peptide hepcidin and its receptor, the iron transporter ferroportin. Hepcidin binds to ferroportin resulting in degradation of ferroportin and decreased cellular iron export. We identify the hepcidin-binding domain (HBD) on ferroportin and show that a synthetic 19 amino acid peptide corresponding to the HBD recapitulates the characteristics and specificity of hepcidin binding to cell-surface ferroportin. The binding of mammalian hepcidin to ferroportin or the HBD shows an unusual temperature dependency with increased rate of dissociation at temperatures below 15°C. The increased rate of dissociation is due to temperature-dependent changes in hepcidin structure. In contrast, hepcidin from poikilothermic vertebrates, such as fish or frog, binds the HBD in a temperature-independent fashion. The affinity of hepcidin for the HBD permits a rapid, sensitive assay of hepcidin from all species and yields insights into the evolution of hepcidin.

INTRODUCTION

Systemic iron homeostasis is dependent on the expression of the liver peptide hormone hepcidin and its interaction with the cell-surface iron transporter ferroportin (Fpn) (for review, see Ganz and Nemeth, 2006). Fpn exports iron from cells to plasma and is responsible for iron absorption from the intestine, recycling of erythrocyte iron by macrophages, and as shown in mice, maternal delivery of iron to the fetus (Donovan et al., 2005). Transcription of hepcidin in hepatocytes is regulated by a variety of stimuli including cytokines (TNF-α, IL-6), erythropoietic activity, iron stores, and hypoxia (De Domenico et al., 2007a). Once secreted, hepcidin binds to Fpn and induces its internalization and degradation (Nemeth et al., 2004). Binding of hepcidin to Fpn leads to the phosphorylation of either of two adjacent tyrosines in a cytosolic domain of Fpn, resulting in the internalization of phosphorylated Fpn by coated pits (De Domenico et al., 2007b). In humans, mutations in Fpn that prevent hepcidin binding or subsequent phosphorylation of Fpn ablate Fpn-hepcidin internalization (De Domenico et al., 2007b). The resultant persistence of cell-surface Fpn leads to a phenotype similar to classical hereditary hemochromatosis, a disorder associated with inappropriately low levels of hepcidin (De Domenico et al., 2006). The difficulty of producing useful anti-hepcidin antibodies has made measurements of plasma hepcidin problematic although measurements have been made by mass spectroscopy (Kemna et al., 2007; Murphy et al., 2007). Here we report the identification of the hepcidin-binding domain (HBD) on Fpn and show that a synthetic peptide corresponding to the HBD can be used in a competitive assay to measure hepcidin concentrations in small volumes of biological fluids. Mammalian hepcidin does not bind to the HBD at low temperature, whereas fish hepcidin binds to the HBD in a temperature-independent manner. This difference in binding activity is correlated with differences in antimicrobial activity and provides insight into the evolution of vertebrate hepcidin.

RESULTS

Identification of the Hepcidin-Binding Site on Fpn

Most of the reported Fpn mutations that lead to hepatic parenchymal iron overload disease in humans do not affect hepcidin binding (De Domenico et al., 2005). There are three Fpn mutations, however, that show no measurable binding of hepcidin (E.N., unpublished data). All three are missense mutations at position C326 (C326Y/T/S). (The number of the amino acid is based on its position in the full-length protein.) This residue is in an extracellular loop, which is just distal to the predicted cytosolic loop containing the two adjacent tyrosines that are phosphorylated in response to hepcidin binding (De Domenico et al., 2007b). We hypothesized that this extracellular loop contained the HBD. To test this hypothesis, we synthesized a 19 amino acid peptide that corresponds to the sequence of the extracellular loop spanning amino acids 324 through 343 and determined if this peptide could compete with cell-surface Fpn for hepcidin binding. Incubation of HEK293TFpn-green fluorescent protein (GFP) cells with hepcidin for 1 hr or 24 hr resulted in the internalization of cell-surface Fpn-GFP (Nemeth et al., 2004), as shown in Figure 1A and quantified in Figure 1B. Preincubation of equimolar amounts of the predicted HBD with

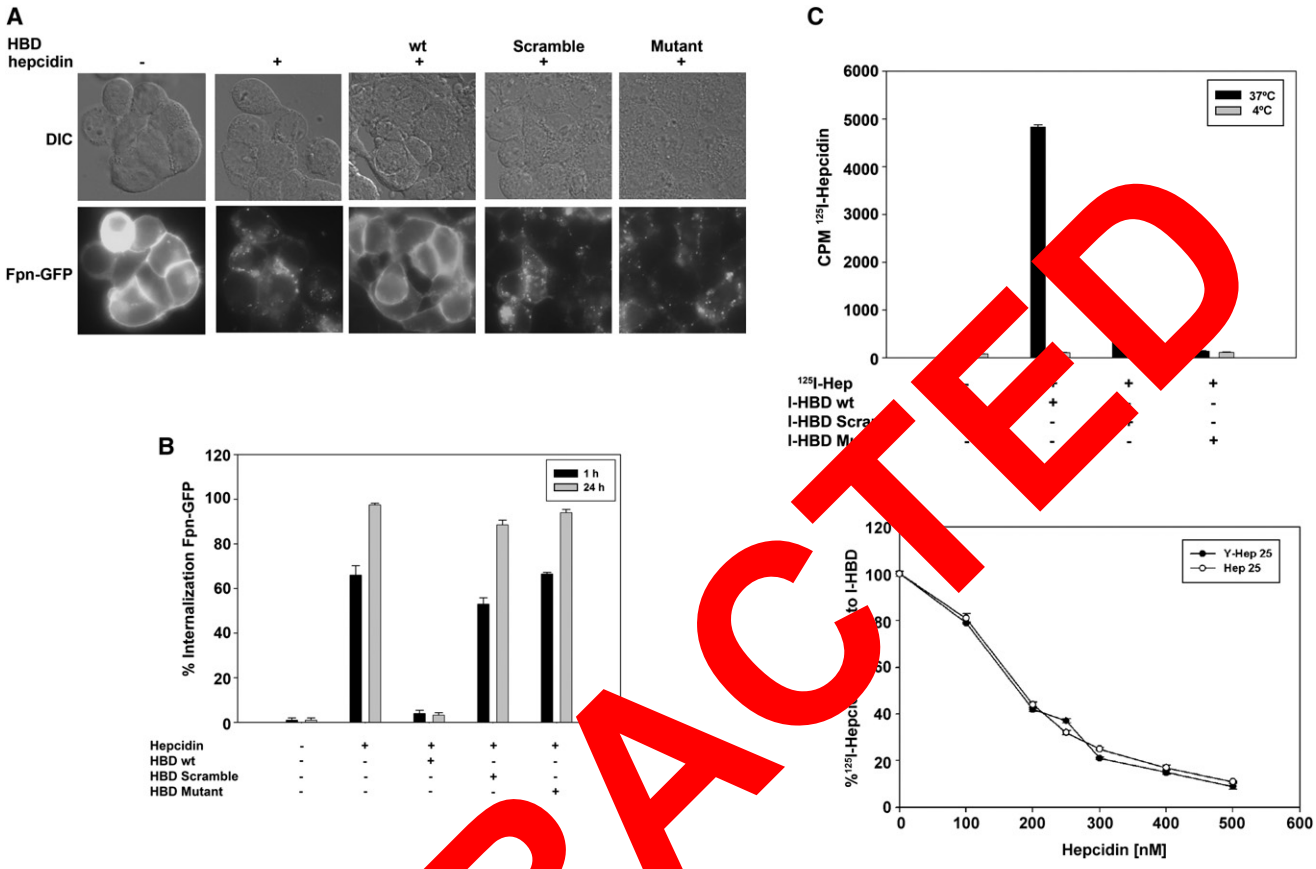

Figure 1. HBD Inhibits Binding of Hepcidin to

(A) HEK293TFpn-GFP cells were incubated or with pcidin (1 μg/ml) or with hepcidin that had been preincubated either with HBD, scrambled HBD, or HBD with the C326Y mutation (HBD mu (The numbe amino acid is based on its position in the full-length protein.) The HBD peptides and hepcidin were mixed at an equimolar ratio for C and then ac to cells for 1 hr or 24 hr. Fpn internalization at 1 hr was analyzed by epifluorescence.

(B) The percentage of cells showing ternal pn-GFP at 1 h and 24 hr was quantified. The data are reported as the standard error of the mean and were determined by counting ten fie containing 2 ells/field.

(C) Peptides (HBD, scramble D, or mutant C32 D) were conjugated to agarose beads (I-HBD). ^{125}I-hepcidin was added to I-HBD for 18 hr at 37°C (black bars) or 4°C (gray bars). beads w re washed, an he amount of ^{125}I-hepcidin bound to beads was determined as counts per minute (cpm).

(D) Specified concent s of he n (Hep25) or hepcidin containing a tyrosine residue (M21Y and Y-Hep25) were added to I-HBD for 1 hr prior to the addition of ^{125}I-hepcidin. The mi w cubated f 18 hr at 37°C, and the amount of ^{125}I-hepcidin bound to beads was determined. The data are expressed as the percentage ^{125}I-hepcidin b o I-HBD xperiments were repeated a minimum of five times, and the error bars represent the standard error of the mean.

hepcidi prev d hep mediated internalization of Fpn- This not due to inhibition of endocytosis, as the amou i rescent dextran was similar in cells incubated or without HBD (data not shown). Preincubation of hepcidin w peptide containing the same amino acid composition as HBD ut in a randomized sequence (HBD scramble) had little effect on Fpn internalization, as did preincubation with an Fpn peptide containing the C326Y mutation (HBD mutant).

To further characterize the interaction of the HBD with hepcidin, the HBD peptide was conjugated to agarose beads (I-HBD) and incubated with ^{125}I-hepcidin at 37°C. As shown previously, ^{125}I-hepcidin was capable of inducing Fpn-GFP internalization (Nemeth et al., 2004). When hepcidin or ^{125}I-hepcidin was preincubated with I-HBD, followed by removal of the I-HBD, the remaining supernatant lost the ability to internalize Fpn-GFP, indicating that I-HBD bound hepcidin (see Figure S1B online). No loss of internalization activity was seen when ^{125}I-hepcidin was preincubated with beads containing either HBD scrambled or C326Y HBD mutant (data not shown). These results indicate that ^{125}I-hepcidin interacts in a specific manner with the HBD even when the HBD is immobilized. The use of ^{125}I-hepcidin permitted a quantitative assay of binding activity. ^{125}I-hepcidin bound only to I-HBD at 37°C and not to the C326Y-HBD mutant and showed severely reduced binding to the HBD scramble (Figure 1C). Accordingly, no loss of internalization activity was seen when ^{125}I-hepcidin was preincubated with I-HBD at 4°C (Figure S1B). The binding of ^{125}I-hepcidin to I-HBD could be competed by simultaneous addition of hepcidin (Hep25). For these experiments, we utilized a M21Y-substituted hepcidin to generate a radioactive molecule. These data show that the M21Y hepcidin binds with identical affinity (apparent K_d of approximately 200 nM) to native hepcidin (Figure 1D).

We took advantage of this assay to examine the importance of amino acids surrounding C326 in Fpn. We synthesized different HBDs in which alanine was substituted for specific amino acids surrounding C326 (Figure 2A). We assayed the effect of those

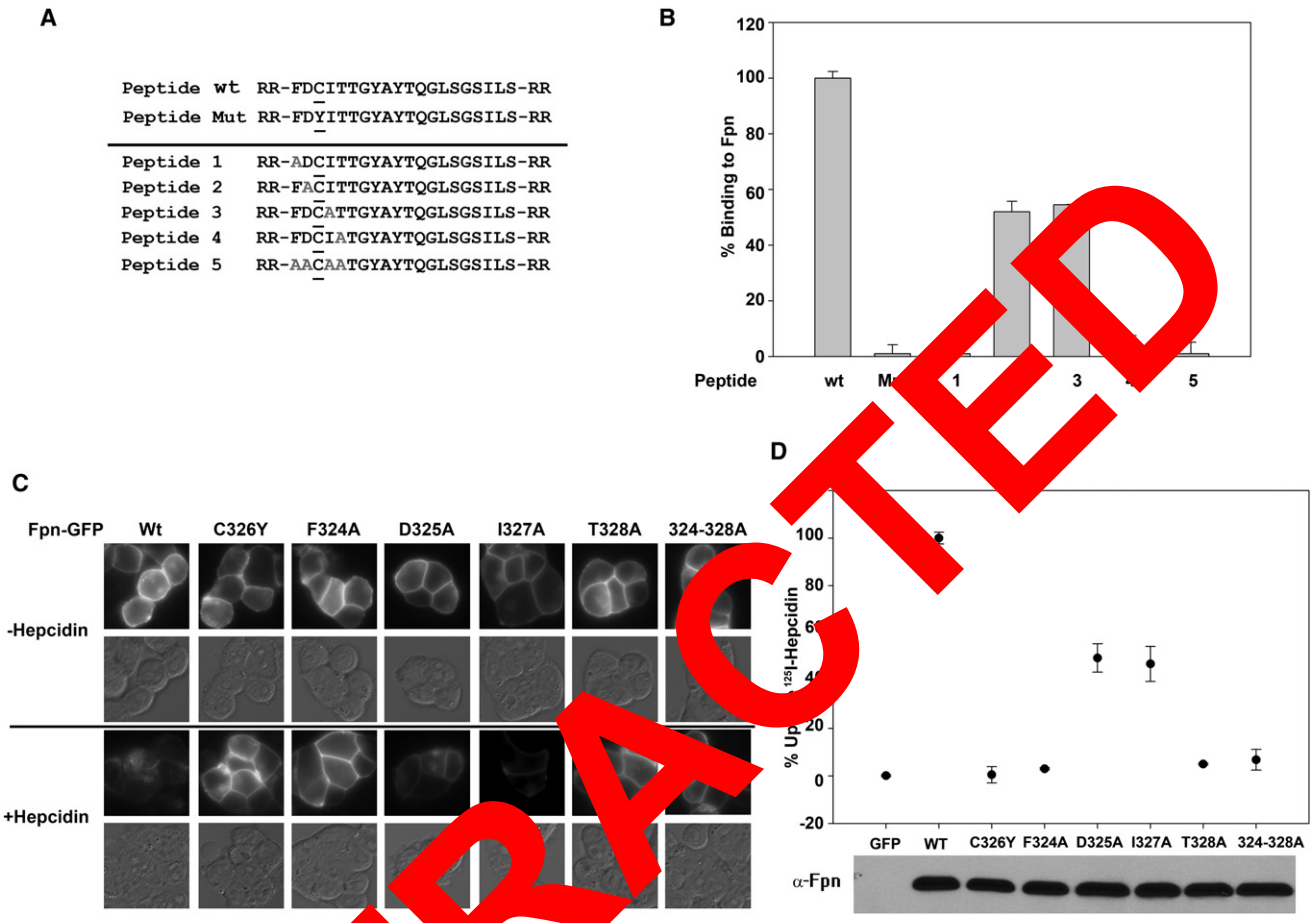

Figure 2. Effect of Amino Acid Substitutions on the Binding of Hepcidin to the HBD and Cell-Surface Fpn

(A) HBDs containing amino acid substitutions (gray alanines) were synthesized.

(B) The ability of modified HBD to bind ^{125}I-hepcidin was assayed as described in Figure 1.

(C) Amino acid substitutions were generated in Fpn-GFP by site-specific mutagenesis as described in Experimental Procedures. Plasmids containing wild-type or mutant Fpn-GFP were transiently transfected into HEK293T cells for 18–24 hr. The transfected cells were incubated with hepcidin (4 μg/ml) for 4 hr, and internalization of Fpn-GFP was assayed by epifluorescence microscopy.

(D) ^{125}I-hepcidin was added to HEK293T cells expressing wild-type Fpn-GFP or mutant Fpn-GFP, and cell-associated radioactivity was measured. The expression of Fpn-GFP constructs was assessed by western-blot analysis using antibodies to Fpn with 25 μg of protein loaded per lane. The data are expressed as percentage uptake in wild-type binding to cells expressing wild-type Fpn-GFP was normalized to 100%. All experiments were repeated a minimum of five times, and the error bars represent the standard error of the mean.

substitutions measuring the ability of the modified HBD to promote hepcidin-mediated internalization of cell-surface Fpn-GFP (Figure S2) and by examining the ability of the modified HBD to bind hepcidin (Figure 2B). All alanine substitutions affected the ability of the HBD to bind hepcidin. It was surprising, however, that the relatively conservative change of A for I on residue 327 significantly affected binding activity (Figures 2B and S2).

To examine the correlation between hepcidin binding to the HBD and hepcidin binding to cell-surface Fpn-GFP, we generated the same alanine substitutions in Fpn-GFP and measured hepcidin-mediated internalization and degradation as well as the binding of ^{125}I-hepcidin to mutant Fpn-GFP-expressing cells. When transfected into HEK293T cells, all mutant Fpn-GFP constructs were expressed at the same level and were appropriately targeted to the cell surface (Figure 2C). Addition of hepcidin led to the internalization and degradation of wild-type Fpn-GFP, but there was little internalization or degradation of Fpn(C326Y)-GFP, Fpn(F324A)-GFP, Fpn(T328A)-GFP, and Fpn(324-328A)-GFP. Hepcidin was able to partially induce the internalization/degradation of Fpn(D325A)-GFP and Fpn(I327A)-GFP. Uptake of ^{125}I-hepcidin to mutant Fpn-GFP cells showed the same pattern: no radioactivity associated with substitutions C326Y, F324A, T328A, and 324-328A, and half-maximal radioactivity was found on cells transfected with Fpn mutants D325A and I327A (Figure 2D). It is noteworthy how well all of the assays (internalization and quantitative hepcidin binding) for the cell-surface mutants and the HBD mutants agree. These results show that binding of hepcidin to the HBD faithfully reproduces binding of hepcidin to cell-surface Fpn.

Binding of Hepcidin to the HBD Is Temperature Sensitive

Previously, we determined that hepcidin binding to Fpn was temperature dependent; binding was detected at 37°C but not at 4°C (Nemeth et al., 2004). Preincubation of HBD with hepcidin

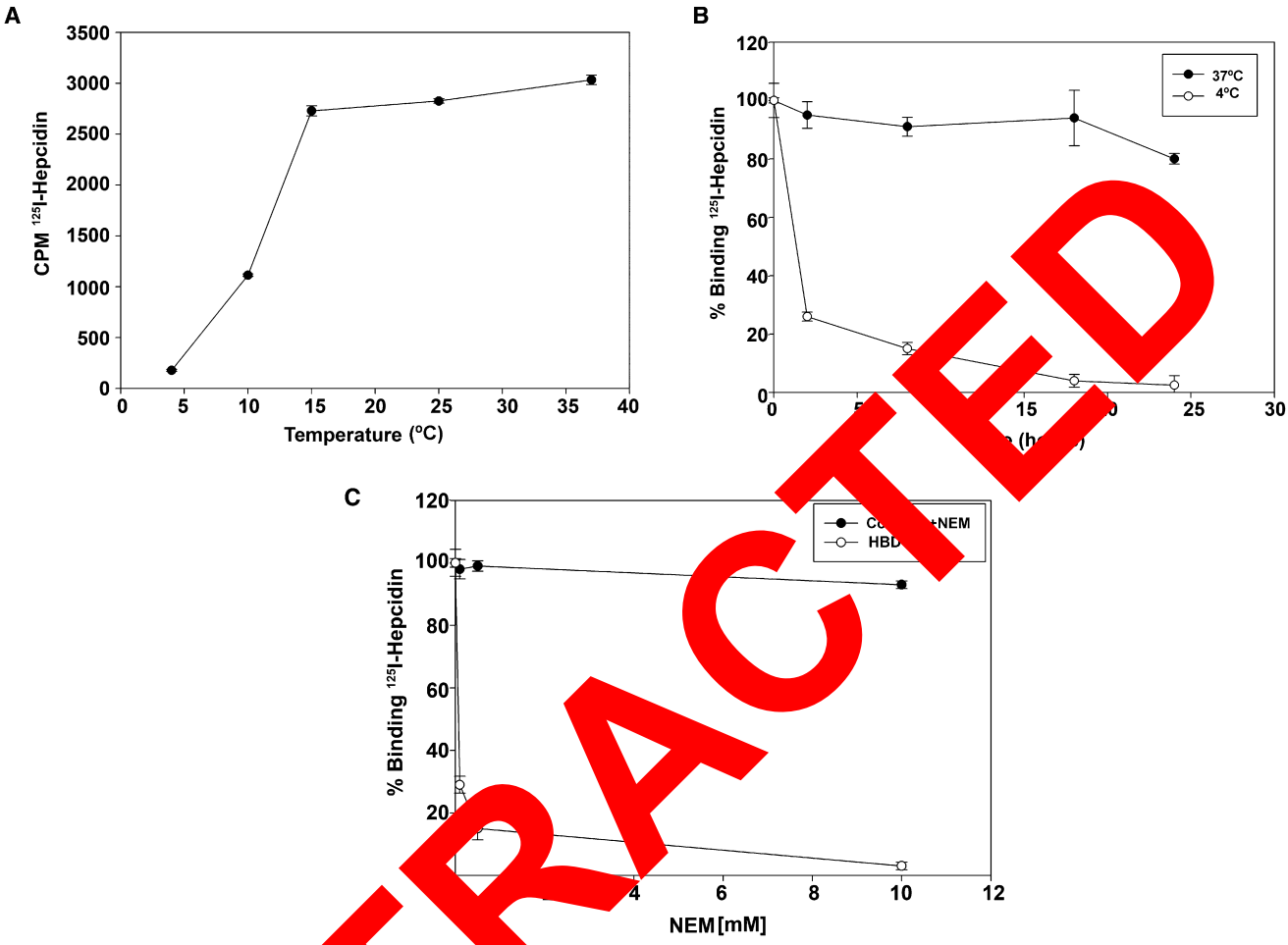

Figure 3. Dissociation of Mam[illegible]an [illegible]idin-HBD Co[illegible]plexes Is Accelerated by Low Temperature

(A) A subsaturating concentrati[illegible] of ^{125}I-hepci[illegible]00 nM) was incubated with I-HBD for 18 hr at the specified temperatures, and the amount of radioactivity bound to beads was determ[illegible]

(B) ^{125}I-hepcidin was adde[illegible]-HBD, and a complex [illegible] allowed to form at 37°C. The beads were washed and incubated with excess nonradioactive hepcidin at 37°C (closed circles) o[illegible] (open [illegible]s) for the specified times, and the amount of radioactivity bound to beads was determined. The data are expressed as radioactivity at each ti[illegible] int[illegible]ve to the amount bound at zero time.

(C) I-HBD was incubated [illegible]erent con[illegible]ations of NEM for 4 hr at 37°C. The beads were washed, ^{125}I-hepcidin was added, and the amount of radioactivity bound to bea[illegible]determi[illegible]open [illegible]es). Alternatively, the I-HBD-^{125}I-hepcidin complex was allowed to form at 37°C. The beads were washed and then incubated [illegible]the s[illegible]fied co[illegible]ns of NEM for 4 hr, and radioactivity bound to the beads was determined (closed circles). The data are expressed as the amount [illegible]dioacti[illegible]t each time [illegible]nt relative to the amount of radioactivity bound to beads prior to NEM treatment. All experiments were repeated a minimum of five [illegible] and [illegible]represent the standard error of the mean.

at 37°C follo[illegible]d by addition of the mixture to cells at 37°C prevented the [illegible]pcidin-mediated internalization of Fpn-GFP, whereas preincubation at 4°C did not (Figure S1). Preincubation of hepcidin and HBD was required, as simultaneous addition of hepcidin and HBD to cells did not prevent internalization of Fpn-GFP (data not shown). Examination of the binding of ^{125}I-hepcidin to I-HBD as a function of temperature showed that binding was decreased dramatically at temperatures below 15°C (Figure 3A). The ^{125}I-hepcidin-I-HBD complex was extremely stable at 37°C. In the presence of a large molar excess of nonradioactive hepcidin, there was little dissociation of preformed ^{125}I-hepcidin-I-HBD over a time course of 24 hr (closed circles in Figure 3B). When the ^{125}I-hepcidin-I-HBD complex was shifted from 37°C to 4°C, ^{125}I-hepcidin rapidly dissociated from the I-HBD (open circles in Figure 3B). Treatment of HBD with the alkylating agent N-ethylmaleimide (NEM) led to a loss of ^{125}I-hepcidin-binding activity (open circles in Figure 3C), suggesting that the free cysteine in the HBD is required for hepcidin binding. NEM added to a preformed ^{125}I-hepcidin-I-HBD complex at 37°C did not result in dissociation of the complex (closed circles Figure 3C). Since NEM inactivates the binding activity of the HBD, we conclude that binding of hepcidin to the HBD prevents access of NEM to the free cysteine in the HBD.

Structural Changes in Hepcidin Affect HBD Binding

To define the mechanism of the temperature sensitivity of hepcidin binding, we examined the properties of hepcidin and HBD by

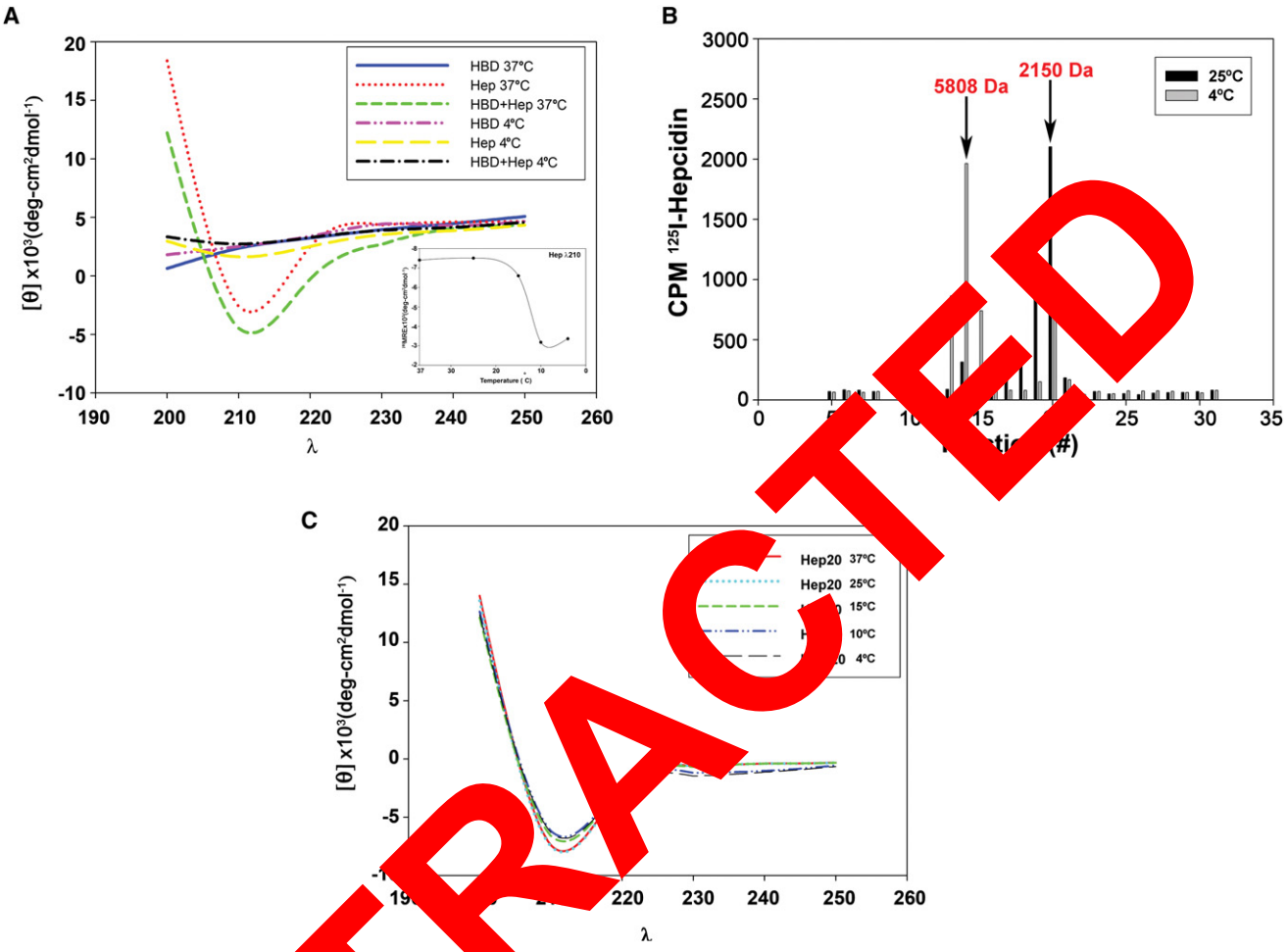

Figure 4. Temperature-Dependent Conformational Changes in Mammalian Hepcidin Structure

(A) Human hepcidin (Hep), HBD, or an equimolar mixture of HBD and hepcidin were analyzed by CD at 37°C and 4°C from wavelength (λ) 190 to 260. The insert shows the effect of temperature on the CD spectrum of hepcidin measured at 210 nm.

(B) ^{125}I-hepcidin was applied to a G-25 column, equilibrated, and eluted at 25°C (black bars) or 4°C (gray bars), and the amount of radioactivity in eluted fractions was determined. The arrows represent the elution of molecular weight standards, insulin (5808 Da), and DBI (2150 Da). The elution of the standards was not affected by temperature.

(C) The CD spectrum of Hep20 (100 μM) was measured at different temperatures. All experiments were repeated a minimum of five times, and the error bars represent the standard error of the mean.

circular dichroism (CD) spectroscopy. Hepcidin exhibited a CD spectrum characteristic of mostly β sheets with a small amount of α-helical structure when measured at 20°C or higher (Figure 4A), a result consistent with published studies (Nemeth et al., 2006). An equimolar mixture of HBD and hepcidin had a CD spectrum similar to that of hepcidin alone. There was a dramatic change in the CD spectrum at 4°C, showing little secondary structure for the hepcidin-HBD mixture or for hepcidin alone. These results indicate that the structure of hepcidin is temperature sensitive. We determined that the change in hepcidin structure occurs between 15°C and 10°C (small insert in Figure 4A), similar to the temperature where hepcidin no longer binds to the HBD.

The temperature-dependent change in hepcidin structure resulted in a change in the multimerization state of hepcidin. Previous studies indicated that hepcidin formed multimers in a concentration-dependent manner (Hunter et al., 2002). At the concentrations employed in this study and at 25°C, hepcidin is a monomer, as shown by size-exclusion chromatography (Figure 4B). When the same preparation of hepcidin was incubated at 4°C and then applied to size-exclusion chromatography, hepcidin eluted predominantly as a dimer.

Derivatives of hepcidin lacking the first five amino acids (Hep20) do not bind to Fpn (Nemeth et al., 2004) nor do they bind to HBD (data not shown). CD analysis of Hep20 showed a spectrum identical to that of hepcidin when assayed at 25°C (Nemeth et al., 2006). In contrast to hepcidin, the CD spectrum of Hep20 did not change when assayed at 4°C (Figure 4C), nor did Hep20 show a temperature-dependent multimerization when assayed by size-exclusion chromatography (data not shown). These results suggest that the temperature-dependent changes in hepcidin structure require the presence of the amino terminal domain of hepcidin.

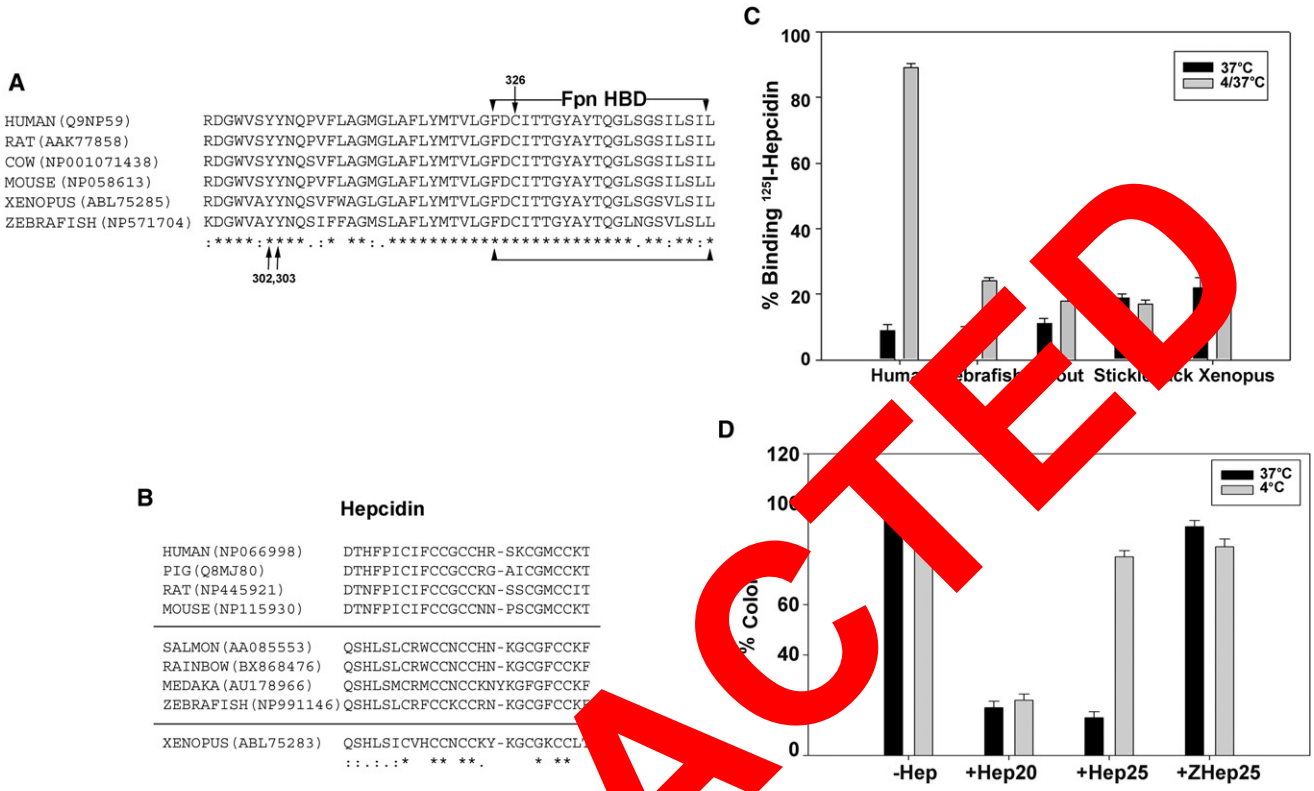

Figure 5. Alignment of Vertebrate Fpn-HBD and nd the ct of Temperature on Hepcidin Activity

(A) Alignment of HBD sequence from homeothermic m blo and poi ermic (cold blooded) vertebrates. The arrows denote the location of the tyrosines that are phosphorylated upon hepcidin bind d cyst 326, which en mutated leads to hepcidin resistance and ferroportin-linked hemochromatosis. The "*" represents identity and the ":" r sion numbers are listed next to the sequence.

(B) Alignment of hepcidin sequence from m als, fis *Xenopus laevis*.

(C) I-HBD was incubated with human or afish hepcidi th serum from *Salmo trutta*, *Pungitius pungitius*, or *Xenopus laevis* for 12 hr at 37°C. The beads were washed three times, and ^{125}I-he added for 1 hr C. The amount of radioactivity bound was determined and compared to that bound to I-HBD, which had been incubated with ^{12} epcidi 1 hr (black bars). An aliquot of I-HBD that had been incubated with hepcidin or sera was washed and then incubated at 4°C for 12 hr. Fol ing washing, epcidin was added at 37°C, and the amount of radioactivity was determined after 1 hr (gray bars).

(D) *E. coli*, grown in LB broth e incubated with 3 f hepcidin (Hep25), Hep20, or zebrafish hepcidin (ZHep25) at 37°C (black bars) or 4°C (gray bars) for 4 hr. Aliquots of bacteria were ed on LB nd incubated a 37°C for 12 hr, and the number of colonies was determined. The data are expressed as the percentage of colonies observed in t sence pcidin. All experiments were repeated a minimum of five times, and the error bars represent the standard error of the mean.

Hepcidin ikil ms es Not Show Tempe re-s sitive g to the HBD

Core perat below 20°C are unusual among mammals, and it mperatures have any specific effect on iron bolism. The core temperature of hibernating mammals (s s ground squirrels) can reach 4°C. At these temperatures, m abolic activity is severely reduced. Many poikilothermic vertebrates such as fish, however, routinely live at temperatures below 15°C, and we questioned whether fish hepcidin would show temperature-sensitive binding to HBD. The sequence of the Fpn HBD in poikilothermic and homeothermic vertebrates shows near identity (Figure 5A). The few instances of sequence variation are highly conserved and are not consistent features of either poikilotherms or homeotherms. Fish appear to have multiple hepcidin genes, which encode hepcidin peptides that are 25, 22, and 20 amino acids in length (for review, see Shi and Camus, 2006). In contrast to the HBD, the sequence of the 25 amino acid form of hepcidin shows consistent differences between poikilotherms and homeotherms (Shi and Camus, 2006). The carboxyl terminal amino acid and the first five amino terminal amino acids are different for the two groups (Figure 5B). Zebrafish hepcidin binds to mammalian Fpn, inducing its internalization and degradation (Nemeth et al., 2006). Chemically synthesized zebrafish hepcidin bound to the HBD at 37°C, and similar-to-human hepcidin showed a slow rate of dissociation at 37°C. Unlike human hepcidin, however, zebrafish hepcidin did not dissociate from the HBD at 4°C (Figure 5C). Similar results were obtained using sera of *Salmo trutta* (brown trout) or *Pungitius pungitius* (Alaskan nine-spine stickleback), fish that live at temperatures below 10°C. Sera from these fish inhibited the binding of ^{125}I-hepcidin to the I-HBD at 37°C. When the serum-incubated I-HBD was placed at 4°C, no binding activity was recovered when assayed at 37°C. This result suggests that hepcidin present in fish serum bound to the I-HBD but did not dissociate at 4°C. Serum from the amphibian *Xenopus laevis* also bound to the HBD in a temperature-independent fashion, suggesting that Hep25 from poikilothermic vertebrates may behave similarly.

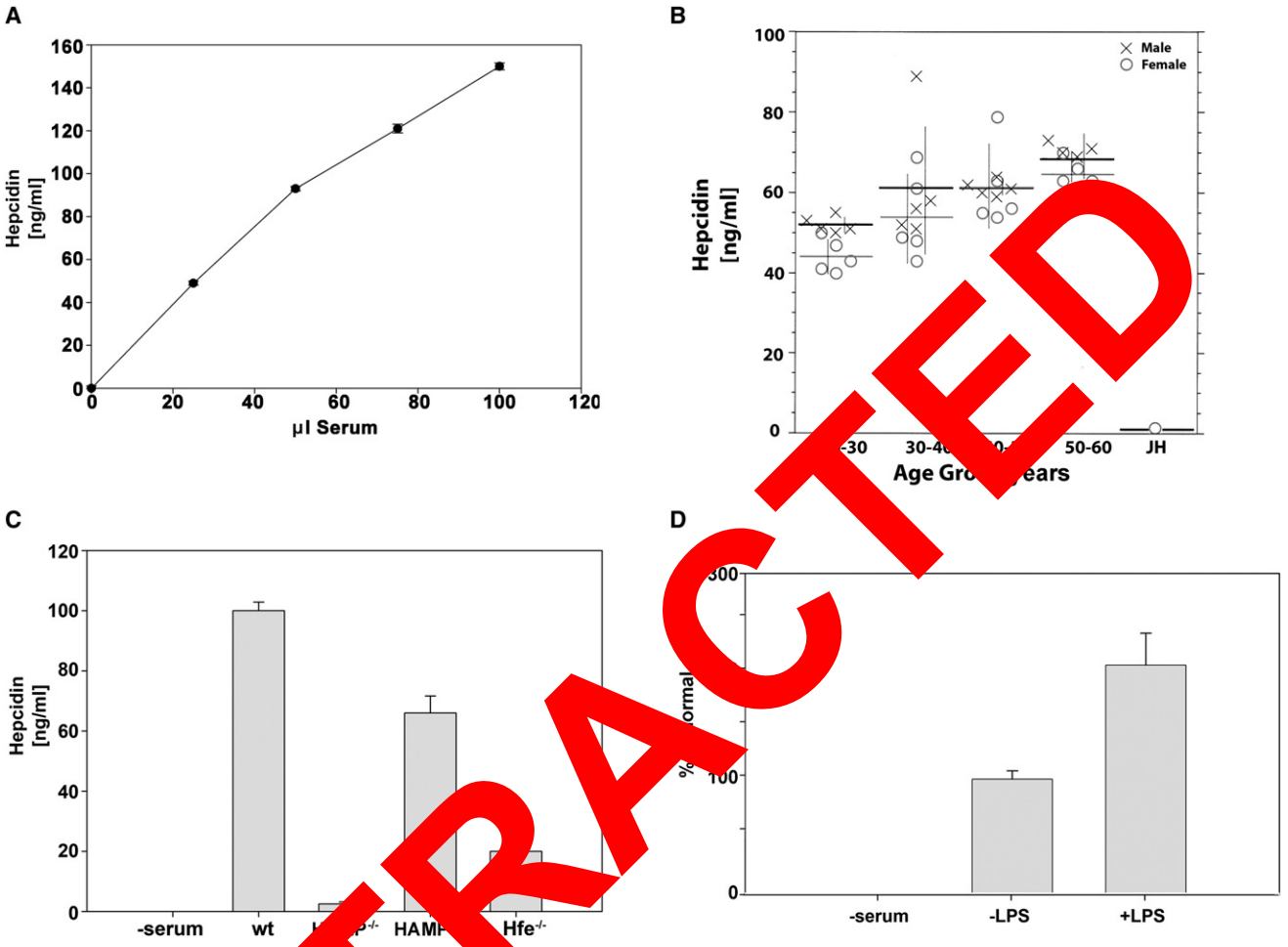

Figure 6. Determination of H[illegible]idin [illegible]entrations in Human and Mouse Sera

(A) Samples (25 µL) from po[illegible]d human sera [illegible] incubated with I-HBD and a known concentration of ^{125}I-hepcidin for 18 hr at 37°C. The beads were then washed, and the amount [illegible]dioactivity was d[illegible]ined. Serum hepcidin levels were calculated relative to a standard curve constructed using chemically synthesized human he[illegible]n. For th[illegible]se and the dat[illegible]escribed below, each sample was measured in triplicate, and the error bars represent the standard error of the mean.

(B) Hepcidin levels w[illegible]te[illegible]d in sera obtained from normal individuals of defined age and gender or in serum obtained from a female patient diagnosed with JH due to mutations in t[illegible] gene as[illegible]cribed in (A).

(C) Hepcidi[illegible]n sera [illegible]ed f[illegible]wild-type mice (C57BL/6), mice homozygous (*HAMP*$^{-/-}$), or heterozygous (*HAMP*$^{+/-}$) for a targeted gene deletion in *HAMP*. [illegible]ice t[illegible]ere hom[illegible]s for a targeted gene deletion in *HFE* were determined as described in (A).

(D) C[illegible]HeJ mic[illegible]ere injected [illegible]traperitoneally with LPS, serum was obtained from the mice 12 hr later, and hepcidin concentration was determined as desc[illegible]in (A)[illegible]xpressed as percentage of normal in which hepcidin levels in the absence of LPS is 100%. All experiments were repeated a minimum o[illegible]es, and th[illegible]rror bars represent the standard error of the mean.

Hepcidin w[illegible]irst identified as an antimicrobial peptide (Park et al., 2001), and we questioned whether hepcidin antimicrobial activity is affected by temperature. Human hepcidin (Hep25) showed temperature-sensitive behavior in its ability to bind to *Escherichia coli*, as measured by binding of ^{125}I-hepcidin to *E. coli* (data not shown) and by its antimicrobial activity (Figure 5D). The antimicrobial activity of Human Hep20 was unaffected by temperature. In contrast to human hepcidin, zebrafish hepcidin (ZHep25) had severely reduced antimicrobial activity against *E. coli* at either temperature. These results suggest that the multiple hepcidin genes found in poikilothermic vertebrates may have evolved to diversify the iron regulatory and antimicrobial activities.

Measurement of Hepcidin in Biological Fluids

We recognized that binding of ^{125}I-hepcidin to I-HBD could be used to assay hepcidin concentrations in biological fluids. We assayed the ability of human serum to compete with ^{125}I-hepcidin for binding to the I-HBD. We determined the concentration of hepcidin in serum and showed a linear relationship with serum volume (Figure 6A). A known quantity of chemically synthesized hepcidin added to serum was quantitatively recovered (data not shown). Repeated measurements of hepcidin in sera showed less than 5.0% variation. We did observe a decrement (10%) in binding activity upon freezing and thawing, but the decrement was independent of absolute hepcidin level. Measurement of hepcidin in sera from a population of normal individuals showed

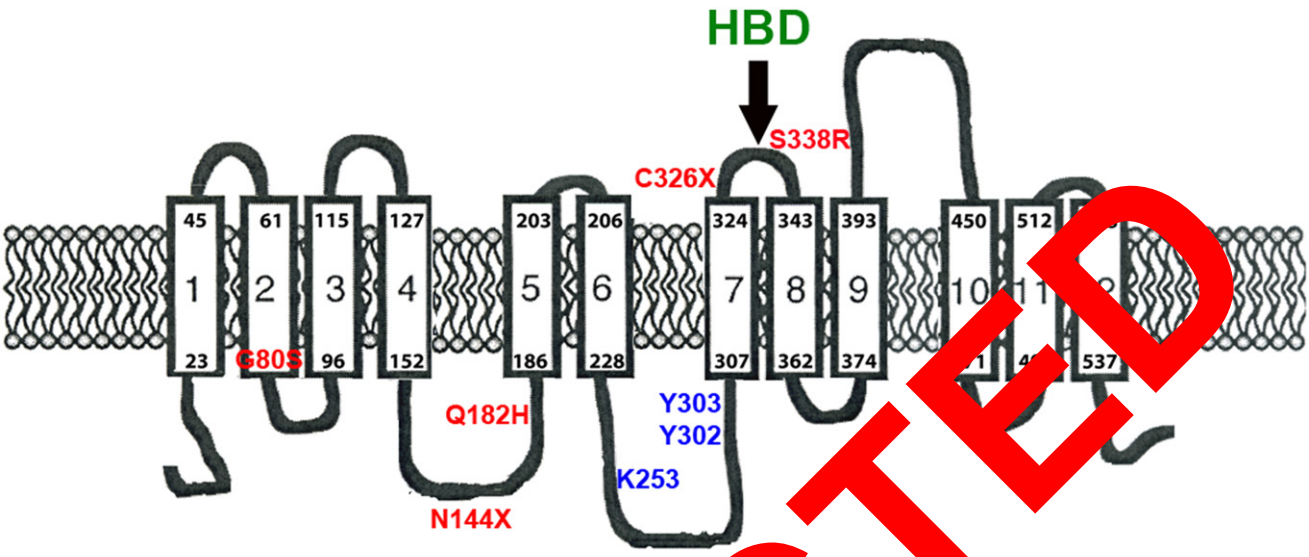

Figure 7. Predicted Topology of Fpn Showing the Position of Mutations that Lead to Hepcidin Resistance

The structure of Fpn is based on the study of Liu et al., 2005. We have added the locations of known human mutations that result in hepcidin-resistant iron overload disease S338R (Wallace et al., 2007), C326S (Sham et al., 2005), N144H (Njajou et al., 2002), Q182H (Hetet et al., 2003), and G80S (De Domenico et al., 2006a). We have also noted the positions of introduced amino acid substitutions that affect hepcidin binding and Fpn internalization (Y302 and Y303) and degradation (K253A) (De Domenico et al., 2007b).

gender-associated differences: males had, in general, higher hepcidin levels than females (Figure 6B). Serum hepcidin levels also showed an age-dependent increase, which is consistent with age-dependent increases in iron burden (Cook et al., 1981). Sera from a population of normal females (20–[illegible] showed an average of 50 ng/ml hepcidin, while an age-matched individual with juvenile hemochromatosis (JH) with mutations in the hemojuvelin gene (*HJV*) (Huang et al., 2004), showed very low levels of hepcidin.

The conservation of hepcidin and its sequences also permitted the assay of hepcidin levels in other vertebrates. We measured the levels of human hepcidin in sera obtained from wild-type mice C57BL/6, mice homozygous ($HAMP^{-/-}$), and heterozygous ($HAMP^{+/-}$) for a targeted deletion in the hepcidin gene (*HAMP*) (Lesbordes-Brion et al., 2006), or mice homozygous ($Hfe^{-/-}$) for a targeted mutation in *HFE*, a gene implicated in hepcidin transcription (Levy et al., 1999) (Figure 6C). Sera obtained from wild-type C57BL/6 mice contained approximately 80–100 ng/ml hepcidin. Sera from $HAMP^{-/-}$ mice had essentially undetectable levels of hepcidin, while $HAMP^{+/-}$ mice showed 70% of normal values. $Hfe^{-/-}$ mice showed lower levels of hepcidin than the $HAMP^{+/-}$ mice. Hepcidin expression increases in response to inflammatory stimuli (Nicolas et al., 2002); Injection of lipopolysaccharide (LPS) into C3H/HeJ mice indeed resulted in increased serum hepcidin (Figure 6D). These data demonstrate that I-HBD can be used to assay hepcidin levels in biological fluids.

DISCUSSION

The identification of the HBD elucidates many features regarding the physiology of hepcidin and Fpn. Binding of hepcidin to Fpn leads to the internalization and degradation of Fpn, preventing cellular iron export, and it is a major controlling factor of systemic homeostasis (Nemeth et al., 2004). The HBD is in the extracytosolic loop of Fpn adjacent to the cytosolic loop containing the two tyrosines required to signal internalization (Figure 7). The figure shows human mutations that lead to hepcidin resistance, which can be ascribed to either an inability to bind hepcidin or an inability to respond to bound hepcidin. There are other human mutations in ferroportin that lead to defective iron export, most commonly because the mutant ferroportins are not appropriately trafficked to the cell surface (for review, see De Domenico et al., 2006b and Liu et al., 2005). To date, only mutations in the HBD have been identified to prevent hepcidin binding. The HBD is separated from the cytosolic loop containing the phosphorylation site by a single transmembrane domain. The juxtaposition of these two domains suggests that even small changes in protein conformation resulting from binding of hepcidin to Fpn might lead to a change in the conformation of the cytosolic loop making the tyrosines accessible to a protein kinase. Three independent mutations in cysteine 326 have been identified in human patients with hepcidin-resistant iron overload disease (Drakesmith et al., 2005; Sham et al., 2005, and E.N., unpublished data). Recently, the mutation S338R—which is in the HBD—has been reported to lead to hepcidin-resistant iron overload disease (Wallace et al., 2007). As shown in this study, amino acid substitutions surrounding cysteine 326 also prevent hepcidin binding. This result suggests that a number of mutations can lead to a functional iron transporter that is correctly targeted to the cell surface but is unable to bind and respond to hepcidin.

Mammalian hepcidin binds to Fpn (Nemeth et al., 2004) or the I-HBD in a temperature-dependent manner. We initially thought that the lack of binding of hepcidin to Fpn-GFP-expressing cells at low temperature reflected weak binding and that the more stable association of hepcidin with cells at 37°C reflected the internalization of the hepcidin-Fpn complex. Our data, however, shows that the lack of binding of hepcidin to Fpn at low

temperature reflects a greatly increased rate of dissociation of the Fpn-hepcidin complex due to a temperature- sensitive change in hepcidin structure. The structure of human hepcidin is suggested to be a hairpin formed from a distorted antiparallel β sheet stabilized by disulfide bonds, and it is predicted to be amphipathic, showing both a hydrophilic and hydrophobic surface (Hunter et al., 2002). Human hepcidin structure is markedly altered at low temperature where hepcidin forms dimers. The change in hepcidin structure is dependent on the amino terminus of hepcidin, as Hep20 does not change its structure or form dimers at low temperature. The change in structure affects its binding to bacteria at low temperature. The five amino terminal amino acids of hepcidin were also previously shown to be critical for binding of hepcidin to Fpn (Nemeth et al., 2006), as Hep20 does not bind to Fpn and, as shown here, does not bind to the I-HBD. The CD structure of Hep20 does not change at low temperature, and the antimicrobial activity of Hep20 is not affected by low-temperature exposure.

Binding of hepcidin to the HBD faithfully reflects binding of hepcidin to cell-surface Fpn. Truncated forms of hepcidin that do not bind to Fpn do not bind to the HBD. Mutations in Fpn and in the HBD affect binding to hepcidin in an identical manner. We have presented data showing that Fpn is a dimer (De Domenico et al., 2006a, 2005). The binding of hepcidin to the HBD, which is monomeric, and to Fpn, which is dimeric, is [illegible]. This suggests that dimer formation does not affect hepcidin binding activity. The one difference we have observed in hepcidin binding is that binding of hepcidin to cell-surface Fpn has a kinetic advantage over binding to the HBD [illegible]C, the rate of dissociation of mammalian hepcidin from the HBD is extremely slow; yet, simultaneous addition of the HBD [illegible] hepcidin to cells did not prevent hepcidin from binding to cell-surface Fpn. Our explanation for this observation is that the "tethering" of the HBD to cell surfaces provides a kinetic advantage in the association constant of binding. A precedent for the kinetic advantage of membrane tethering was shown years ago through studies of cytochrome b5 (Enoch et al., [illegible]).

There is a high degree of conservation among vertebrate hepcidins from both homeothermic and poikilothermic species. Most mammals have a single functional hepcidin gene, in contrast to fish, which have multiple hepcidin genes (Shi and Camus, 2006). Fish appear to have at least one hepcidin gene that encodes a mature form of 25 amino acids, whereas many of the other genes encode mature hepcidins of less than 25 amino acids. Based on studies of zebrafish and mammalian hepcidins, these smaller hepcidin forms should not bind to Fpn. Most fish hepcidins have a similar amino terminus, and hepcidin from tropical (zebrafish) and cold water (brown trout, stickleback) fish occupy the HBD at both high and low temperatures. The binding of fish hepcidin to Fpn regardless of temperature indicates that it regulates iron metabolism.

There are many studies on the expression of fish hepcidins, but there is only limited evidence for the antimicrobial activity for the different-sized hepcidins (Lauth et al., 2005; Shike et al., 2002). As shown here, the zebrafish 25 amino acid hepcidin has little antibacterial activity against *E. coli*. A chemically synthesized 25 amino acid hepcidin (TH2-3) from the fish Tilapia had antimicrobial activity against some bacteria, whereas a 23 amino acid Tilapia hepcidin (TH1-5) had antimicrobial activity against different bacteria (Huang et al., 2007). A third putative Tilapia hepcidin (TH2-2) had no antimicrobial activity against the tested bacteria. Together, these results suggest that mammalian hepcidin has both iron regulatory and antimicrobial activity, while fish hepcidin genes have evolved to separate these two functions. A recent study has shown that fish hepcidins, in contrast to mammalian hepcidin, have been under increased selective pressure, as shown by the presence of multiple hepcidin genes and by increased mutations in non-synonymous positions (Padhi and Verghese, 2007). Mammalian sequences only appear to contain the mature 25 amino acid form of hepcidin, but 22 and 20 amino acid hepcidins are found in humans (Park et al., 2001). It had been noted that the 20 amino acid hepcidin had greater antimicrobial activity than the 25 amino acid form (Park et al., 2001). We confirmed this observation using *E. coli*. We suggest that the generation of the smaller forms may not affect the catabolism of hepcidin but [illegible] the generation of a more potent antibacterial hepcidin.

Finally, our data indicate that the I-HBD can be used to assay hepcidin in biological fluids. The values we report are similar to the values reported for the measurement of hepcidin in mouse serum using mass spectroscopy (Murphy et al., 2007). The values we obtained for the average concentration of human hepcidin in serum (35–70 ng/ml), however, are higher than those reported in human serum by the mass-spectroscopy measurements (Murphy et al., 2007; Tomosugi et al., 2006). Human and mouse hepcidin are nearly identical, as are human and mouse Fpn. It is unclear why the steady concentration of serum hepcidin in mouse and human might be different. Our data indicate that the steady-state levels of human and mouse hepcidin are fairly similar. The HBD assay detects biologically active (with respect to Fpn binding) hepcidin. It does not detect hepcidins that do not bind to Fpn, such as Hep20. The HBD assay does not detect urinary hepcidin. The inability to detect urinary hepcidin does not result from an interference of urine with the assay, as the assay can readily measure hepcidin added to human urine (data not shown). We believe that hepcidin present in urine is not biologically identical to serum hepcidin. Support for this view comes from a mass-spectroscopic analysis, which suggests the 25 amino acid form of hepcidin present in human urine has a lower molecular mass than serum hepcidin (Kemna et al., 2005).

We present data showing that the HBD assay can readily detect variations in serum hepcidin levels due to mutations in genes known to affect hepcidin levels (*HAMP*), mutations in other genes involved in iron metabolism (*HFE*), or LPS stimulation. It is interesting to note that mice heterozygous for a deletion in *HAMP* only show a 30% reduction in serum hepcidin, suggesting that some allelic compensation might be occurring. This result is consistent with the relatively normal iron levels and phenotype found in $HAMP^{+/-}$ mice. Most mammals have one hepcidin gene, while mice have a second highly homologous gene termed *HAMP2* (Pigeon et al., 2001). The expression of both *HAMP1* and *HAMP2* were regulated by iron (Ilyin et al., 2003). Expression of *HAMP2* as a transgene had no effect on iron metabolism (Lou et al., 2004), although mRNA levels for *HAMP2* are induced by inflammatory stimuli. The observation that mice with a targeted deletion in *HAMP* have essentially no Fpn-binding activity in their sera indicate that the *HAMP2* protein does not interact with Fpn, which explains its lack of effect on iron metabolism. The

sensitivity of the I-HBD hepcidin assay offers a facile approach to determining hepcidin levels in serum as well as other biological fluids. As demonstrated here, the evolutionary conservation of the HBD permits measurements of hepcidin in the serum of all vertebrates.

EXPERIMENTAL PROCEDURES

Cells and Media

HEK293T Fpn, a stable cell line in which Fpn-GFP expression is regulated by the ecdysone promoter, was described previously (Nemeth et al., 2004).

Generation of Fpn Constructs

All human-Fpn mutations were generated in pFpn-EGFP-N1 by using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene) amplified in *E. coli*, and it was sequence verified before transfecting into mammalian cells, as described in De Domenico et al., 2005.

Peptide Synthesis

Peptides corresponding to wild-type and mutant HBDs were synthesized at the DNA/Peptide Core Facility, University of Utah, Salt Lake City, Utah. To increase solubility, the peptides were synthesized with two arginines added at the amino and carboxyl termini. The arginines did not affect the specificity, temperature dependence, or affinity of the HBD for hepcidin. Human and zebrafish hepcidin were prepared as described previously (Nemeth et al., 2006). The sequence of the HBD is RR-FDCITTGYAYTQGLSGSILS-RR. The sequence of the scrambled HBD is RR-ILSLFDAYCTGTQITGSGSY-RR. The sequence of the C326Y mutant is RR-FDYITTGYAYTQGLSGSILS-RR.

Peptide Immobilization and Hepcidin-Competition Assay

HBD peptides were immobilized by covalently linking them to agarose beads using AminoLink Plus Immobilization Kit (Pierce) according to manufacturer's instructions (1.0 mg of Fpn peptide per 2.0 ml of amino link beads). To measure hepcidin concentrations in biological samples, samples were mixed with a defined concentration of ^{125}I-hepcidin and the peptide-conjugated beads. The samples were incubated at 4°C overnight for 12–18 hr. The beads were washed multiple times with phosphate buffered saline (PBS) to remove unbound ^{125}I-hepcidin by centrifugation for 2 minutes at 500 × *g*. The fluid was removed, and the radioactivity bound to the beads was determined by gamma counting (Perkin/Elmer).

CD Spectroscopy

CD spectra (200–250 nm) of 100 μM hepcidin, hepcidin (Hep20, which lacks the five amino terminal residues), and HBD in 50 mM sodium phosphate buffer (pH 7.2) were recorded on a model 62DS spectrometer (AVIV, Lakewood, NJ). Samples were analyzed at various temperatures (37°C, 25°C, 15°C, 10°C, and 4°C) using 0.5 mm path-length cells (Starna Cells, CA). Before analysis, spectra were baseline corrected by subtracting spectra of sample-free buffer solutions from the corresponding sample.

Other Procedures

Hepcidin with a C326Y substitution was synthesized, iodinated, and used in binding assays as described (Nemeth et al., 2004). For gel-filtration chromatography, ^{125}I-hepcidin was applied to a G-25 superfine column (Amersham Pharmacia) equilibrated in PBS. The column was calibrated using insulin (5808 Da, Sigma), diazepam-binding inhibitor (DBI) fragment 51–70 human (2150 Da, Sigma), and neurotensin (1672 Da, Sigma) as standards. Fractions (200 μL) were collected and analyzed for ^{125}I-hepcidin by gamma counting (Perkin/Elmer), and SDS-PAGE analysis followed by Coomassie blue staining (Pierce) for standards. The antibacterial activity of different forms of hepcidin was tested for antimicrobial activity against *E. coli* (DH5α). Hepcidin was added to a final concentration of 30 μM at 37°C and 4°C to bacterial cultures grown in LB broth. The cultures were incubated with constant shaking for 4 hr at either 4°C or 37°C. The cells were washed and aliquots were plated on LB plates and incubated at 37°C for measurement of colony-forming units.

All animal studies were approved by the Animal Research Committee at the University of Utah. C3H/HeJ mice were injected with LPS (Sigma) and sacrificed 10 hr later. Animals were euthanized, and blood was collected by cardiac puncture. Mouse sera were collected in accordance with the Institutional Animal Care and Use Committee protocols. Human sera were obtained according to protocols approved by the University of Utah Institutional Review Board and in accordance with the Declaration of Helsinki with informed consent for samples obtained from all subjects. Sera were obtained from *Pungitius pungitius* by Dr. Michael Shapiro (University of Utah) and from *Salmo trutta* harvested on the Middle Provo River using a 22 blue-winged olive, according to the rules of the Utah Department of Wild Life. Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL). Western blot analysis was performed as described previously using mouse raised anti-Fpn (1:1000) followed by peroxidase-conjugated goat anti-rabbit IgG (1:12,500, Jackson ImmunoResearch). Microscopy analysis was carried out using an Olympus BX51 microscope (Olympus, Tokyo, Japan). Images were acquired using Picture Frame 2.5 software (Olympus America, Inc., Muskogee, OK). All experiments were repeated in animals five times, and error bars represent standard error of the mean.

SUPPLEMENTAL DATA

Supplemental Data include figures and can be found online at http://www.cellmetab.org/cgi/content/full/8/2/146/DC1/.

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